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Reproductive and lifestyle factors and circulating sRANKL and OPG concentrations in women: Results from the EPIC cohort

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Running title: Correlates of circulating sRANKL and OPG in women

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Abbreviations:

BMI	Body mass index
DKFZ	German Cancer Research Center
EPIC	European Prospective Investigation into Cancer and Nutrition
ELISA	Enzyme-linked immunosorbent assay
IARC	International Agency for Research on Cancer
LLOD	Lower limit of detection
OC	Oral contraceptive
OPG	Osteoprotegerin
PMH	Postmenopausal hormones
sRANKL	soluble Receptor Activator of Nuclear Factor κB

Abstract

Background: Except for a documented increase in osteoprotegerin (OPG) concentrations with older age, data on determinants of soluble Receptor Activator of Nuclear Factor κ B (sRANKL) and OPG concentrations in women are limited. We evaluated reproductive and lifestyle factors as potential sources of variation in circulating sRANKL and OPG concentrations in pre- and postmenopausal women.

Methods: This study includes 2016 controls (n=1552 (76%) postmenopausal, n=757 (38%) using postmenopausal hormone therapy (PMH)) from a breast cancer case-control study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Serum sRANKL was measured using an enzyme-linked immunosorbent assay, serum OPG using an electrochemiluminescent assay. Generalized linear models were used to evaluate associations between these analytes and reproductive and lifestyle factors.

Results: Older age at blood collection was associated with lower sRANKL concentrations in postmenopausal women ($p_{\text{trend}} \leq 0.03$) and higher OPG concentrations in all women ($p_{\text{trend}} \leq 0.01$). Longer duration of OC use among premenopausal women and postmenopausal PMH users was associated with higher OPG ($p_{\text{trend}} \leq 0.04$). In postmenopausal non-PMH users, sRANKL concentrations were lower with longer duration of OC use and current (vs. never) smoking ($p \leq 0.01$). sRANKL concentrations were higher among women with higher BMI ($p_{\text{trend}} \leq 0.01$). The evaluated factors accounted for 12% of the variation in sRANKL concentrations and 21% of the variation in OPG concentrations.

Conclusion: Circulating sRANKL and OPG concentrations are minimally impacted by hormone-related factors in pre- and postmenopausal women.

Impact: This study suggests circulating concentrations of sRANKL and OPG are unlikely to be strongly modified by hormone-related reproductive and lifestyle factors.

Introduction

The Receptor Activator of Nuclear Factor κ B (RANK) axis includes the receptor, its ligand (RANKL) and the decoy receptor for RANKL, osteoprotegerin (OPG). Studies in experimental animal models have shown that the RANK-axis plays an important role in a number of processes including bone turnover, immune response, cardiovascular disease, and breast development in pregnancy, as well as development and progression of breast cancer [1-3]. With respect to breast cancer, we [4-6] and others [7-10] have observed associations between circulating concentrations of RANK-axis members and breast cancer risk and prognosis. Following development of an antibody for RANKL, denosumab, there is increasing interest in RANKL and OPG in relation to breast cancer prevention and treatment.

Given the relevance of the RANK-axis for a variety of outcomes, understanding whether concentrations of sRANKL (the soluble homotrimeric form of RANKL found in circulation) and OPG are potentially modulated by lifestyle and reproductive factors is of interest. Characterizing these associations contributes toward informing mechanistic understanding of associations between these factors and disease risk, providing an indication as to whether a subgroup of women may have particularly high concentrations due to lifestyle and/or reproductive-related factors, and elucidating whether circulating concentrations may be modifiable via changes in lifestyle and reproductive-related exposures. However, data to date on correlates of sRANKL and OPG concentrations in healthy individuals are limited. In a study of twins, genetic factors explained half of the variation in sRANKL concentrations, whereas OPG concentrations were almost entirely determined by environmental factors including age [11]. A positive correlation between age and OPG concentrations has been reported by a number of studies [11-14], including our own (e.g., Spearman correlation=0.29) [5]. We have also previously reported that OPG concentrations were higher among ever users of oral contraceptives (OCs) [5]. Given interest in circulating OPG and sRANKL for multiple disease outcomes, and following our prior observation of differences in OPG [5] and sRANKL [6] concentrations depending on postmenopausal hormone (PMH) use at blood collection, we conducted a detailed evaluation of associations between sRANKL and OPG concentrations and hormone-related reproductive and lifestyle factors in women by menopausal status and PMH use at blood collection.

Materials and methods

Study population

The participants included in this study were women selected as controls in a case-control study on sRANKL and OPG concentrations and breast cancer risk (n=2023) nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Data on reproductive, lifestyle and anthropometric characteristics were collected for EPIC participants at baseline. Detailed descriptions of data collection with the EPIC cohort and design and baseline characteristics of the parent case-control study have been described in detail previously [5, 6, 15]. For the current study, eligible women were selected among cohort members who donated a baseline blood sample and were alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of their index case. Premenopausal women were eligible if they were not using exogenous hormones at baseline.

Laboratory assays

Serum concentrations of sRANKL and OPG were analyzed at the laboratory of the Division of Cancer Epidemiology at the German Cancer Research Center (DKFZ). Total OPG concentrations were measured using an electrochemiluminescence assay (MesoScale Diagnostics, USA), a volume-efficient alternative to an enzyme-linked immunosorbent assay (ELISA). Free (i.e. non-OPG bound, or bioavailable) sRANKL concentrations were measured using an ELISA (Biomedica, Austria). The majority of sRANKL in circulation is bound to OPG and free sRANKL concentrations are known to be low [16]; free sRANKL concentrations in our study were below the lower limit of detection of the assay (LLOD) in 152 women and were set to 50% of the LLOD (0.01 picomole per liter (pmol/L)). Due to equipment failure two batches (38 women) of samples were not measured for sRANKL. A total of 4 women were missing sRANKL and/or OPG concentrations, leaving a total of 1981 women with sRANKL and 2019 women with OPG measured (**Figure 1**). Inter-batch coefficients of variation (CVs) were 15.6% for premenopausal and 13.3% in postmenopausal women for sRANKL and 16.4% and 16.8%, respectively, for OPG. Intra-batch CVs were 0.9% and 1.5% for sRANKL in pre- and postmenopausal women, and 9.0% and 21.7% for OPG.

Statistical analyses

Outliers were evaluated using the extreme studentized deviate test [17]; three women with outlying OPG concentrations (two with high concentrations and one with low concentrations) were identified and excluded (**Figure 1**). No sRANKL outliers were identified. Means of natural

log-transformed sRANKL and OPG concentrations were compared between categories of reproductive and lifestyle factors using adjusted generalized linear models. These results were exponentiated, providing the geometric mean in the original scale. Confidence limits were calculated subtracting/adding the standard error multiplied by 1.96 (corresponding to the α for a two-sided p value of 0.05) from the geometric mean. P values for differences in concentrations across categories were calculated using Type III sum of squares; p_{trend} was calculated by modelling the continuous variable as the exposure.

We evaluated age at blood collection (<50, 50-54, 55-59, 60+ years), age at menarche (<12, 12-14, 15+ years), oral contraceptive use (never, <5, 5-9, 10+ years), number of term pregnancies (none, 1, 2, 3+) and age at first term pregnancy among women reporting ≥ 1 pregnancies (<25, 25+ years), body mass index (BMI; <20, 20-24.9, 25-29.9, 30+ kg/m²), smoking status (never, former, current) and duration of smoking among ever smokers (≤ 10 , 11-20, 21-30, 31-40, 41+ years), and total physical activity (inactive, moderately inactive, moderately active, active). The total physical activity variable includes household, recreational and occupational activity, as previously described [18, 19]. Additional variables of interest were ever breastfeeding and duration of breastfeeding among parous women reporting ever breastfeeding (<3, 3-6, 6-12, 12+ months), lifetime average alcohol consumption (never drinker, former drinker, >0-6, >6-12, >12 grams/day), and fasting status at blood collection (<3, 3-6, 7+ hours). All analyses adjusted for the matching factors from the parent case-control study: country (as a proxy for study center), age (continuous), menstrual cycle phase (premenopausal women; early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing; estimated using “backward dating” counting backward from start of menses following blood collection where data were available, otherwise estimated using “forward dating” as time since last menses (described in detail in [20]), fasting status (<3, 3-6, 7+ hours, missing), and time at blood collection (continuous), except when the variable was the exposure of interest. In a sensitivity analysis, OC use and smoking status were mutually adjusted. Given our previous observation that sRANKL concentrations were lower [6], and OPG concentrations higher [5], among postmenopausal women using PMH, models were stratified by menopausal status and PMH use at blood collection. OPG and sRANKL concentrations did not vary by menstrual cycle phase in premenopausal women [5, 6]. We estimated the proportion of variance explained by the epidemiological factors using the linear regression model R-squared value.

Inclusion of participants with sRANKL values below the LLOD of the assay led to a non-normal distribution of sRANKL concentrations; these subjects were excluded in a sensitivity analysis. All statistical tests were two-tailed and significant at $p < 0.05$. Statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Lower circulating sRANKL concentrations were observed with older age among women postmenopausal at blood collection ($p_{\text{trend}} \leq 0.03$; **Table 1**). There was no difference in concentrations by age among premenopausal women ($p_{\text{trend}} 0.20$); however, the range in age was limited in this subgroup. In postmenopausal women not using PMH, both ever use of OCs and longer duration of OC use among ever users were associated with lower sRANKL concentrations ($p \leq 0.01$). Among postmenopausal PMH users who ever used OCs, longer duration of use was suggestively inversely associated with sRANKL concentrations ($p_{\text{trend}} 0.07$). Parity ($p \geq 0.18$), and ages at menarche ($p_{\text{cat}} \geq 0.14$) and first full term pregnancy ($p_{\text{cat}} \geq 0.19$) were not associated with sRANKL concentrations.

A higher BMI was associated with higher sRANKL concentrations regardless of menopausal status and PMH use at blood collection ($p_{\text{trend}} \leq 0.01$; **Table 2**). In postmenopausal women not using PMH at blood collection, there was suggestion of lower sRANKL concentrations in current smokers than in never smokers ($p_{\text{cat}} 0.09$; **Table 2**); among women using PMH at blood collection current smokers had significantly lower sRANKL concentrations relative to never smokers ($p_{\text{cat}} 0.01$). The associations between OC use and smoking and sRANKL concentrations were robust to mutual adjustment (data not shown). We found no association between alcohol consumption and sRANKL concentrations ($p \geq 0.22$).

Overall, the epidemiological factors found to be associated with circulating sRANKL concentrations (plus the matching factors) explained 12% of the variability in this analyte in the full population; each individual variable explained $< 6\%$ of the variation in sRANKL concentrations. Results were unchanged after excluding participants with sRANKL concentrations below the LLOD (**Supplemental tables 1 and 2**).

OPG concentrations were highest among women with older age at blood collection ($p_{\text{trend}} \leq 0.01$; **Table 3**). Longer duration of OC use was associated with higher OPG concentrations in premenopausal women ($p_{\text{trend}} 0.04$) and postmenopausal women using PMH ($p_{\text{trend}} < 0.01$) in

analyses including never OC users. The association in premenopausal women was no longer statistically significant after adjustment for smoking ($p_{\text{trend}} = 0.06$; data not tabled), while the association was robust to this adjustment in postmenopausal women using PMH ($p_{\text{trend}} < 0.01$). No significant trend was observed in analyses restricted to ever users of OCs. Parity ($p_{\text{cat}} \geq 0.13$) and ages at menarche ($p_{\text{cat}} = 0.16$) and first full term pregnancy ($p_{\text{cat}} \geq 0.11$) were not associated with OPG concentrations (**Table 3**); neither were any of the investigated lifestyle factors ($p \geq 0.09$; **Table 4**). Overall, the epidemiological factors found to be associated with circulating OPG concentrations (plus the matching factors) explained 21% of the variability. The majority of this variability in OPG concentrations (18%) was explained by age.

In further analyses fasting status ($p_{\text{trend}} \geq 0.15$), breastfeeding history ($p_{\text{cat}} = 0.17$), and menstrual cycle phase ($p_{\text{cat}} \geq 0.18$) did not influence sRANKL or OPG concentrations (**Supplemental tables 3, 4, and 5**). Among premenopausal parous women who breastfed, a longer duration of breastfeeding was associated with lower sRANKL concentrations ($p_{\text{trend}} \leq 0.03$; **Supplemental tables 3 and 4**).

Discussion

We provide a large-scale cross-sectional study on reproductive and lifestyle factors in relation to sRANKL and OPG concentrations in healthy women. Our study suggests that circulating concentrations of these biomarkers are not strongly impacted by these characteristics. This study extends our prior work, where we reported Spearman correlations between sRANKL and OPG concentrations and circulating sex hormones, and age and BMI [5, 6]; here we evaluated geometric mean concentrations for an extensive selection of epidemiologic characteristics, and assessed the total variance explained by these characteristics, to inform future studies incorporating these analytes.

RANKL is highly expressed in bone, lung and lymph nodes and is found at lower levels in tissues including the placenta and heart [21]. Circulating sRANKL is cleaved from RANKL expressed in tissue by metalloproteinases or formed by alternative messenger ribonucleic acid (mRNA) splicing [3]. The RANK-axis is an essential mediator of progesterone-induced proliferation in the breast during pregnancy in preparation for the formation of a lactating gland [22, 23]. In addition to progesterone, prolactin and parathyroid hormone-related peptide, but not estradiol, have been shown to induce RANKL expression in mouse mammary tissue [24-28]. While the main sources of circulating OPG appear to be bone marrow and vascular endothelial

cells [29, 30], it is also produced by osteoblasts and production of OPG in the latter may be upregulated by estrogen and vitamin D, but not progesterone [13, 30-34].

OPG concentrations are known to increase with age [12, 13, 35], which was confirmed in our study. In line with our findings, weak inverse associations between age and sRANKL have been reported [6, 36, 37], with Spearman correlations $< |0.13|$ observed in the current study population [6]; however, the associations observed between age and sRANKL concentrations in prior studies are not entirely consistent and weak positive [38, 39] and null findings [36] have also been reported. Longer duration of OC use was associated with lower concentrations of sRANKL, and higher concentrations of OPG; the associations for sRANKL and for OPG among postmenopausal women not using PMH were robust to adjustment for smoking, while the OPG associations for premenopausal women were attenuated (p 0.06 after adjustment; p 0.04 before adjustment). Prior studies have reported positive associations between current OC use and OPG concentrations, unadjusted for smoking, but no association for sRANKL [40, 41]. To our knowledge there are no prior studies on duration of OC use and circulating sRANKL or OPG. It is plausible prolonged exposure to estrogens in OCs could increase OPG concentrations (and in turn lower free sRANKL concentrations), yet a mechanistic study did not observe changes in serum sRANKL or OPG in macaque monkeys after estrogen and progesterone treatment [42]. Among the relatively small subset of parous women who ever breastfed ($n=332$), longer duration of breastfeeding was associated with lower sRANKL concentrations. While the RANK-RANKL signaling is essential for breast development in pregnancy, we are not aware of any studies describing the role of the RANK-axis in prolonged breastfeeding.

We observed higher concentrations of sRANKL with higher BMI, regardless of menopausal status at blood collection. While Uemura et al. observed no association between sRANKL and BMI in a population of postmenopausal women [39], others have reported a positive association with BMI in populations of men and women [43] and adolescents [44], as well as positive correlations between sRANKL and obesity-related markers of inflammation and insulin resistance [43]. In line with previous studies [12, 39, 45, 46], OPG concentrations were similar across BMI categories in the current study. Our finding of an inverse association between current smoking and sRANKL concentrations among postmenopausal women using PMH, and duration of smoking and sRANKL concentrations among postmenopausal women not using PMH is in agreement with prior studies in women [40] and a population of men and women [47].

No associations were observed between any of the investigated reproductive factors and sRANKL or OPG concentrations. For sRANKL in particular, this may be reflective of weak associations between concentrations in the breast and circulating concentrations, as demonstrated by a previous study in macaques [42]. In addition, we have shown that, though concentrations of OPG were reproducible over time (Spearman r for serum samples taken fourteen years apart: 0.75), long-term reproducibility of sRANKL was lower (Spearman r : 0.38). This indicates one measurement of sRANKL may not reflect longer-term exposure [5, 6]. While we observed no associations between hormone-related factors and circulating sRANKL and/or OPG among women predominantly older than age 50 in the current study, it is plausible that these reproductive factors may impact concentrations in the shorter term, but may not be evident in the longer term, following these reproductive events.

At the time we initiated our study, there were no previous studies on sRANKL or OPG and breast cancer, which was the focus of the parent study. Three studies published since used ELISA assays to measure OPG concentrations [7, 8, 42]. The two studies that report OPG concentrations observed different ranges of OPG concentrations in their study populations: 0.46-25.81 ng/ml (23.12-1296.98pmol/L; [7]) and 4.2-547.7ng/mL (211.06-27522.61pmol/L; [8]), however, the two studies used different ELISAs. Both of these reported concentrations are higher than the range of concentrations we observed in the current study (3.54-33.02pmol/L). Given the lack of a cross-assay standardization protocol, comparison between studies relies on relative within-study differences in concentrations. The ELISA we used to quantify free sRANKL concentrations in our study is the most sensitive assay currently commercially available, but median free sRANKL concentrations observed in our study were relatively low (0.11 pmol/L) and 7.5% ($n=152$) of the study population had concentrations below the LLOD of the assay. One previous study used the same assay to measure sRANKL, but did not report mean or median concentrations, or if any samples were measured to be below the LLOD [9]. Total serum sRANKL concentrations may be over 1000pmol/L, but the majority of sRANKL found in circulation is bound to OPG [16] and cannot bind to RANK to activate signaling. Thus, even though free sRANKL concentrations are low, they are likely a more informative measure than total sRANKL concentrations.

A further limitation to our study is the relatively high inter-batch CVs, especially for OPG concentrations in postmenopausal women, indicating potential measurement error that may have attenuated findings. In addition, one small previous study suggests sRANKL and OPG

concentrations may decrease over time in serum samples stored at temperatures higher than -70°C [48]. Serum samples in our study were stored at -150°C and we observed no correlation between sRANKL and OPG concentrations and date of blood collection (Spearman $r \leq 0.03$; samples collected between 1992 and 2002). R squared values indicate that the evaluated factors explain relatively little of the variation in sRANKL and OPG concentrations. However, this may be influenced by the limited variability in the distributions of the examined factors in our population of middle to older aged European women. Finally, due to multiple comparisons, some of our results may be due to chance.

In this large cross-sectional study, we demonstrate that circulating sRANKL and OPG concentrations are minimally impacted by hormone-related factors in women. In the context of the limited evidence to date, these results suggest circulating concentrations of sRANKL and OPG are unlikely to be modifiable by lifestyle or reproductive factors.

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Ethics approval and consent to participate: This project was approved by the International Agency for Research on Cancer (IARC) Ethics Committee (Project No. 12-42) and the University of Heidelberg Ethics Commission (Project No. S311/2014). The EPIC study protocol was approved by the ethical committees of IARC and the participating centers. All participants provided informed consent.

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Availability of data and material: For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at <http://epic.iarc.fr/access/index.php>

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Table 1. Cross-sectional associations between reproductive characteristics and sRANKL concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection.

	Premenopausal women		Postmenopausal women			
	n (%) ^a	Geometric mean (95%CI)	No PMH		PMH	
			n (%) ^a	Geometric mean (95%CI)	n (%) ^a	Geometric mean (95%CI)
Full study population*	463 (100%)	0.07 (0.06-0.08)	760 (100%)	0.08 (0.07-0.10)	758 (100%)	0.06 (0.05-0.08)
Age at blood collection, years						
<50	370 (80%)	0.10 (0.08-1.13)	12 (2%)	0.09 (0.05-0.17)	31 (4%)	0.10 (0.06-0.17)
50-55	90 (19%)	0.08 (0.06-1.12)	123 (16%)	0.10 (0.07-0.12)	194 (26%)	0.08 (0.06-0.10)
50-60	3 (1%)	‡	279 (37%)	0.08 (0.06-0.09)	309 (41%)	0.07 (0.05-0.09)
60+			346 (46%)	0.07 (0.06-0.09)	224 (30%)	0.06 (0.04-0.08)
P _{trend} ^b		0.20		<0.01		0.03
Age at menarche, years						
<12	80 (17%)	0.11 (0.08-0.15)	101 (13%)	0.09 (0.07-0.12)	120 (16%)	0.06 (0.04-0.08)
12-14	321 (69%)	0.10 (0.08-0.12)	489 (64%)	0.08 (0.07-0.10)	484 (64%)	0.07 (0.06-0.09)
≥15	59 (13%)	0.08 (0.06-0.11)	159 (21%)	0.07 (0.05-0.09)	146 (19%)	0.07 (0.05-0.09)
P _{trend} ^b		0.16		0.14		0.93
Oral contraceptive (OC) use						
Never	165 (37%)	0.10 (0.08-0.13)	473 (65%)	0.08 (0.07-0.10)	282 (41%)	0.07 (0.05-0.09)
< 5 years	157 (35%)	0.09 (0.07-0.12)	158 (18%)	0.09 (0.07-0.11)	164 (24%)	0.09 (0.06-0.12)
5-9 years	75 (17%)	0.10 (0.07-0.14)	50 (7%)	0.07 (0.05-0.10)	78 (11%)	0.06 (0.04-0.09)
≥ 10 years	49 (11%)	0.07 (0.05-0.10)	79 (11%)	0.06 (0.04-0.07)	169 (24%)	0.07 (0.05-0.09)
P _{trend} ^c including never users		0.08		<0.01		0.28
P _{trend} ^{bd} among ever users		0.34		0.01		0.07
Full-term pregnancy (FTP)						
Never	56 (12%)	0.10 (0.07-0.14)	95 (13%)	0.08 (0.06-0.10)	89 (12%)	0.06 (0.04-0.08)
Ever	401 (87%)	0.10 (0.08-0.13)	663 (87%)	0.08 (0.06-0.10)	647 (85%)	0.07 (0.05-0.09)
1 FTP	85 (21%)	0.10 (0.07-0.14)	109 (16%)	0.07 (0.05-0.09)	120 (19%)	0.06 (0.04-0.08)
2 FTPs	217 (54%)	0.10 (0.07-0.14)	308 (46%)	0.08 (0.06-0.10)	316 (49%)	0.07 (0.06-0.10)
≥ 3 FTPs	98 (24%)	0.09 (0.07-0.13)	246 (37%)	0.08 (0.06-0.10)	210 (32%)	0.07 (0.05-0.10)
P _{cat} ^c ever/never		0.77		0.76		0.21
P _{cat} ^{ce} number		0.90		0.60		0.18
Age at first FTP^e						
< 25 years	192 (48%)	0.10 (0.07-0.13)	308 (46%)	0.08 (0.06-0.10)	369 (57%)	0.07 (0.05-0.09)
≥ 25 years	207 (52%)	0.10 (0.07-0.13)	353 (53%)	0.07 (0.06-0.09)	272 (42%)	0.07 (0.05-0.09)
P _{trend} ^b		0.87		0.30		0.19

^a percentages may not add up to 100% due to missing data; ^b P value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables; ^c P value for difference in geometric mean concentration of OPG across categories of the exposure variable. Assessed using type III F statistics from linear regression models; ^d among those who ever used OCs; ^e Among those who ever had a FTP; ‡ Not evaluated; Generalized linear models adjusted for: age (continuous), fasting status (<3, 3-6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing), and country. * P value comparing pre- to postmenopausal at blood collection=0.91; comparing PMH to no PMH use at blood collection <0.01.

Table 2. Cross-sectional associations between lifestyle characteristics and sRANKL concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection.

	Premenopausal women		Postmenopausal women			
	n (%) ^a	Geometric mean (95%CI)	No PMH		PMH	
			n (%) ^a	Geometric mean (95%CI)	n (%) ^a	Geometric mean (95%CI)
Baseline BMI						
< 20 kg/m ²	29 (6%)	0.07 (0.05-0.11)	48 (6%)	0.06 (0.04-0.09)	64 (8%)	0.06 (0.04-0.09)
20-24.9 kg/m ²	252 (54%)	0.09 (0.07-0.11)	322 (42%)	0.07 (0.06-0.09)	426 (56%)	0.06 (0.04-0.08)
25-29.9 kg/m ²	137 (30%)	0.11 (0.08-0.14)	285 (38%)	0.08 (0.07-0.11)	215 (28%)	0.09 (0.07-0.12)
≥ 30 kg/m ²	45 (10%)	0.13 (0.09-0.19)	105 (14%)	0.09 (0.07-0.12)	53 (7%)	0.10 (0.07-0.15)
P _{trend} ^b		<0.01		0.01		<0.01
Baseline smoking status						
Never smoker	245 (53%)	0.10 (0.08-0.13)	435 (57%)	0.08 (0.07-0.10)	395 (52%)	0.08 (0.06-0.10)
Former smoker	110 (52%)	0.10 (0.07-0.13)	178 (56%)	0.08 (0.06-0.10)	199 (59%)	0.06 (0.05-0.08)
Current smoker	103 (48%)	0.08 (0.06-0.11)	140 (44%)	0.07 (0.05-0.09)	138 (41%)	0.05 (0.04-0.08)
≤ 10 years ^c	45 (21%)	0.12 (0.08-0.19)	41 (13%)	0.08 (0.05-0.13)	55 (16%)	0.05 (0.03-0.08)
11-20 years ^c	62 (29%)	0.12 (0.08-0.18)	44 (14%)	0.05 (0.03-0.07)	50 (15%)	0.06 (0.03-0.11)
21-30 years ^c	87 (41%)	0.10 (0.07-0.15)	67 (21%)	0.08 (0.05-0.11)	73 (22%)	0.08 (0.05-0.13)
31-40 years ^c	13 (6%)	0.07 (0.04-0.14)	90 (28%)	0.07 (0.05-0.11)	95 (28%)	0.04 (0.03-0.07)
> 40 years ^c	0	‡	58 (18%)	0.06 (0.04-0.09)	41 (12%)	0.05 (0.03-0.10)
P _{cat} ^d never vs. current smoking		0.15		0.09		0.01
P _{trend} ^{bc} duration of smoking		0.15		0.65		0.58
Baseline physical activity						
Inactive	75 (16%)	0.09 (0.07-0.12)	80 (11%)	0.07 (0.05-0.10)	148 (20%)	0.07 (0.05-0.10)
Moderately inactive	145 (31%)	0.11 (0.08-0.14)	241 (32%)	0.07 (0.06-0.09)	319 (42%)	0.07 (0.06-0.10)
Moderately active	193 (42%)	0.09 (0.06-0.11)	381 (50%)	0.08 (0.07-0.10)	231 (30%)	0.07 (0.05-0.09)
Active	50 (11%)	0.09 (0.06-0.12)	58 (8%)	0.07 (0.05-0.10)	59 (8%)	0.07 (0.04-0.10)
P _{cat} ^c inactive vs. active		0.95		0.89		0.62
Lifetime alcohol consumption						
Non-drinker	40 (9%)	0.10 (0.07-0.16)	79 (10%)	0.08 (0.06-0.11)	27 (4%)	0.06 (0.03-0.10)
Former drinker	15 (3%)	0.08 (0.04-0.14)	41 (5%)	0.05 (0.04-0.08)	12 (2%)	0.07 (0.03-0.14)
Current drinker	395 (87%)	0.10 (0.08-0.14)	639 (81%)	0.08 (0.06-0.10)	696 (94%)	0.07 (0.05-0.09)
< 6 gram/day ^e	144 (32%)	0.10 (0.07-0.14)	216 (28%)	0.08 (0.06-0.10)	181 (24%)	0.07 (0.05-0.09)
6-12 gram/day ^e	168 (37%)	0.11 (0.08-0.15)	295 (39%)	0.08 (0.06-0.11)	337 (45%)	0.07 (0.05-0.10)
≥12 gram/day ^e	83 (18%)	0.09 (0.06-0.13)	110 (14%)	0.08 (0.06-0.10)	179 (24%)	0.06 (0.05-0.09)
P _{cat} ^d never vs. current alcohol use		0.94		0.86		0.46
P _{trend} ^b gram/day ^e		0.81		0.87		0.53

^a percentages may not add up to 100% due to missing data; ^b P value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables; ^c Among current and former smokers; ^d P value for difference in geometric mean concentration of sRANKL across categories of the exposure variable. Assessed using type III F statistics from linear regression models; ^e among current drinkers; ‡ Not evaluated; Generalized linear models adjusted for: age (continuous), fasting status (<3, 3-6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing), and country.

Table 3. Cross-sectional associations between reproductive characteristics and OPG concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection.

	Premenopausal women		Postmenopausal women			
	n (%) ^a	Geometric mean (95%CI)	No PMH		PMH	
			n (%) ^a	Geometric mean (95%CI)	n (%) ^a	Geometric mean (95%CI)
Full study population*	464 (100%)	9.48 (9.08-9.91)	795 (100%)	9.80 (9.48-10.14)	757 (100%)	10.25 (9.88-10.63)
Age at blood collection, years						
<50	370 (80)	8.11 (7.64-8.60)	13 (2%)	8.55 (7.36-9.92)	31 (4%)	8.60 (7.80-9.49)
50-54	91 (19)	9.38 (7.75-9.10)	127 (16%)	8.80 (8.29-9.35)	194 (26%)	9.36 (8.86-9.88)
55-59	3 (1)	‡	292 (37%)	9.70 (9.18-10.24)	308 (41%)	9.31 (8.82-9.82)
60+			363 (46%)	10.84 (10.30-11.40)	224 (30%)	10.60 (10.01-11.23)
P _{trend} ^b		0.01		<0.01		<0.01
Age at menarche						
<12 years	80 (17%)	7.84 (7.28-8.45)	104 (13%)	10.21 (9.54-10.93)	120 (16%)	9.49 (8.93-10.10)
12-14 years	322 (70%)	8.14 (7.68-8.63)	517 (66%)	9.89 (9.42-10.37)	483 (64%)	9.76 (9.27-10.27)
≥15 years	59 (13%)	8.22 (7.59-8.90)	163 (21%)	10.11 (9.53-10.71)	146 (19%)	9.78 (9.19-10.41)
P _{trend} ^b		0.18		0.23		0.16
Oral contraceptive (OC) use						
Never	166 (37%)	7.88 (7.39-8.40)	495 (65%)	9.92 (9.45-10.41)	282 (41%)	9.34 (8.83-9.89)
< 5 years	157 (35%)	8.18 (7.68-8.72)	134 (18%)	10.13 (9.52-10.78)	164 (24%)	9.56 (8.99-10.16)
5-9 years	75 (17%)	8.19 (7.59-8.85)	51 (7%)	9.60 (8.81-10.47)	78 (11%)	9.70 (9.01-10.45)
≥ 10 years	47 (11%)	8.46 (7.77-9.22)	81 (11%)	10.23 (9.50-11.01)	168 (24%)	10.15 (9.53-10.81)
P _{trend} ^c including never users		0.04		0.64		<0.01
P _{trend} ^{bd} among ever users		0.38		0.86		0.07
Full-term pregnancy (FTP)						
Never	57 (12%)	7.98 (7.33-8.69)	102 (13%)	10.34 (9.69-11.04)	88 (12%)	9.53 (8.90-10.20)
Ever	401 (86%)	8.15 (7.66-8.67)	691 (87%)	9.92 (9.47-10.39)	647 (85%)	9.72 (9.26-10.21)
1 FTP	85 (21%)	8.24 (7.57-8.97)	110 (16%)	9.53 (8.91-10.21)	120 (19%)	9.78 (9.17-10.43)
2 FTPs	216 (54%)	8.13 (7.55-8.75)	317 (46%)	10.03 (9.51-10.58)	316 (49%)	9.64 (9.15-10.17)
≥ 3 FTPs	99 (25%)	8.33 (7.71-8.99)	264 (38%)	9.88 (9.35-10.44)	210 (32%)	9.68 (9.14-10.25)
P _{cat} ^c ever/never FTP		0.54		0.13		0.46
P _{cat} ^{ce} number of FTP		0.66		0.22		0.86
Age at first FTP^e						
< 25 years	191 (48%)	8.30 (7.72-8.92)	318 (46%)	9.83 (9.33-10.36)	369 (57%)	9.74 (9.24-10.27)
≥ 25 years	208 (52%)	8.03 (7.46-8.64)	371 (54%)	9.98 (9.45-10.54)	272 (42%)	9.85 (9.32-10.40)
P _{trend} ^b		0.11		0.26		0.65

^a percentages may not add up to 100% due to missing data; ^b P value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables; ^c P value for difference in geometric mean concentration of OPG across categories of the exposure variable. Assessed using type III F statistics from linear regression models; ^d among those who ever used OCs; ^e Among those who ever had a FTP; ‡ Not applicable; Generalized linear models adjusted for: age (continuous), fasting status (<3, 3-6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing), and country. *P value comparing pre- to postmenopausal at blood collection=0.57; comparing PMH to no PMH use at blood collection <0.01.

Table 4. Cross-sectional associations between lifestyle characteristics and OPG concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection.

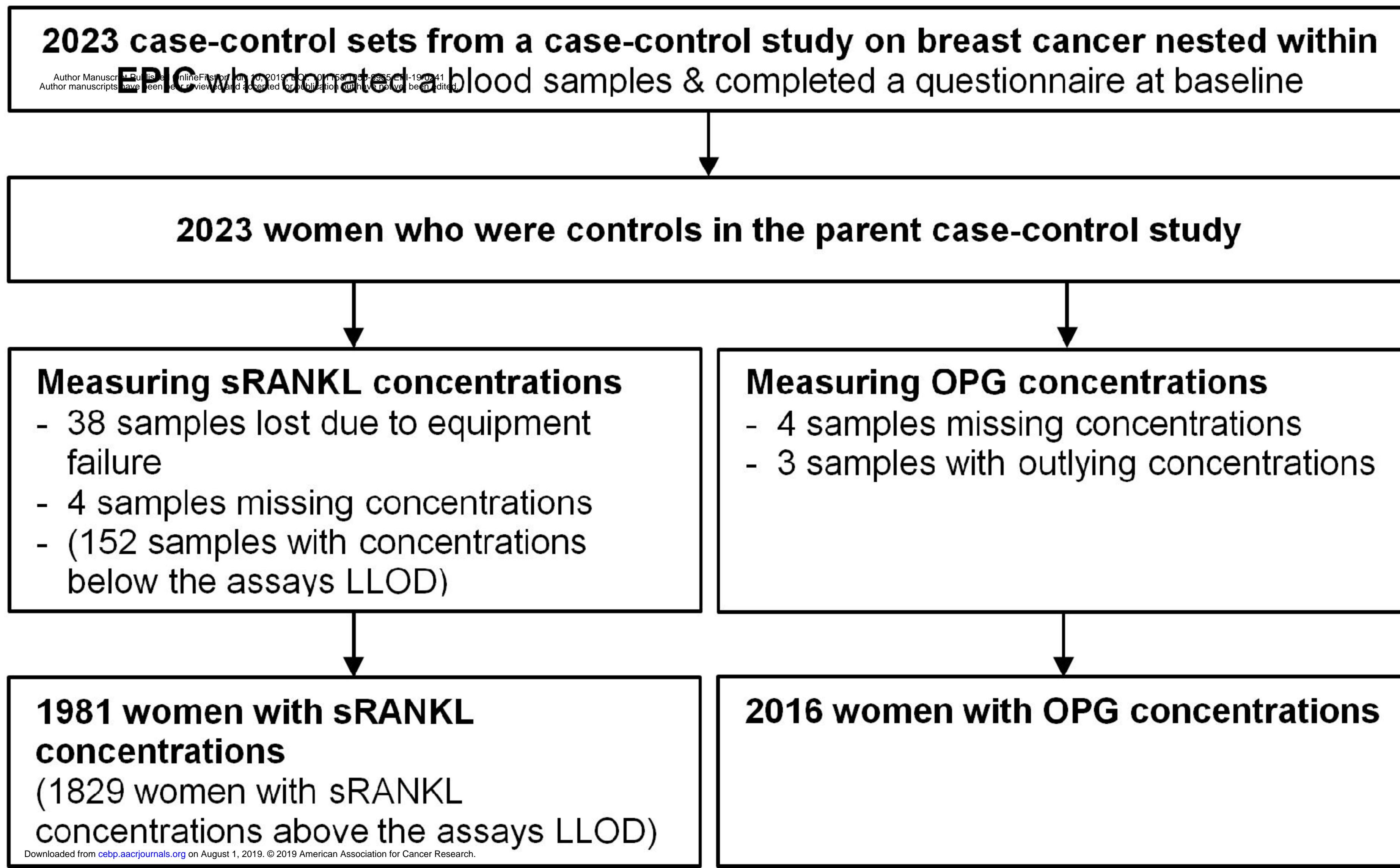
	Premenopausal women		Postmenopausal women			
	n (%) ^a	Geometric mean (95%CI)	No PMH		PMH	
			n (%) ^a	Geometric mean (95%CI)	n (%) ^a	Geometric mean (95%CI)
Baseline BMI						
< 20 kg/m ²	29 (6%)	7.76 (7.01-8.59)	48 (6%)	10.18 (9.35-11.09)	64 (8%)	10.31 (9.55-11.12)
20-24.9 kg/m ²	252 (54%)	8.17 (7.69-8.67)	336 (42%)	9.98 (9.49-10.51)	426 (56%)	9.72 (9.23-10.23)
25-29.9 kg/m ²	138 (30%)	8.10 (7.59-8.66)	299 (38%)	9.82 (9.32-10.35)	214 (28%)	9.612 (9.09-10.17)
≥ 30 kg/m ²	45 (10%)	8.09 (7.41-8.83)	112 (14%)	10.16 (9.53-10.82)	53 (7%)	9.41 (8.69-10.19)
P _{trend} ^b		0.74		0.60		0.23
Baseline smoking status						
Never smoker	247 (53%)	8.00 (7.53-8.50)	463 (58%)	9.94 (9.47-10.44)	395 (52%)	9.70 (9.21-10.22)
Former smoker	110 (52%)	8.18 (7.66-8.75)	181 (56%)	9.88 (9.33-10.45)	198 (59%)	9.62 (9.09-10.17)
Current smoker	102 (48%)	8.40 (7.83-9.00)	144 (44%)	10.27 (9.65-10.93)	138 (41%)	9.95 (9.35-10.59)
≤ 10 years ^c	45 (21%)	7.73 (7.04-8.49)	42 (13%)	9.88 (8.75-11.15)	55 (16%)	9.91 (8.93-11.01)
11-20 years ^c	62 (29%)	7.86 (7.18-8.60)	44 (14%)	10.44 (9.24-11.78)	50 (15%)	9.23 (8.25-10.31)
21-30 years ^c	86 (41%)	8.41 (7.73-9.16)	68 (21%)	10.03 (9.04-11.12)	73 (22%)	9.45 (8.54-10.46)
31-40 years ^c	13 (6%)	7.41 (6.41-8.57)	92 (28%)	10.97 (9.88-12.19)	94 (28%)	10.07 (9.11-11.14)
> 40 years ^c	0	†	60 (18%)	9.90 (8.84-11.08)	41 (12%)	10.43 (9.22-11.81)
P _{cat} ^d never vs. current smoking		0.09		0.20		0.33
P _{trend} ^{bc} duration of smoking		0.36		0.59		0.20
Baseline physical activity						
Inactive	75 (16%)	8.08 (7.49-8.72)	82 (10%)	9.82 (9.12-10.57)	148 (20%)	9.47 (8.89-10.10)
Moderately inactive	145 (31%)	8.03 (7.54-8.56)	247 (31%)	9.88 (9.34-10.44)	319 (42%)	9.75 (9.21-10.31)
Moderately active	193 (42%)	8.39 (7.86-8.94)	405 (51%)	10.00 (9.53-10.50)	230 (30%)	9.77 (9.26-10.30)
Active	51 (11%)	8.86 (7.26-8.52)	61 (8%)	10.25 (9.48-11.09)	59 (8%)	9.57 (8.87-10.34)
P _{cat} ^d inactive vs. active		0.52		0.33		0.78
Lifetime alcohol consumption						
Non-drinker	41 (9%)	8.04 (7.28-8.87)	88 (11%)	10.64 (9.87-11.47)	27 (4%)	10.40 (9.35-11.57)
Former drinker	15 (3%)	7.99 (6.95-9.18)	49 (6%)	11.13 (10.16-12.19)	12 (2%)	10.01 (8.67-11.55)
Current drinker	395 (85%)	8.11 (7.55-8.70)	639 (80%)	10.25 (9.69-10.84)	696 (93%)	9.87 (9.36-10.40)
< 6 gram/day ^e	144 (31%)	8.23 (7.60-8.90)	219 (28%)	10.13 (9.52-10.78)	181 (24%)	10.01 (9.42-10.64)
6-12 gram/day ^e	168 (36%)	8.00 (7.42-8.62)	305 (38%)	10.36 (9.75-11.02)	337 (45%)	9.74 (9.21-10.30)
≥12 gram/day ^e	83 (18%)	8.18 (7.53-8.89)	115 (14%)	10.27 (9.57-11.02)	178 (24%)	9.95 (9.36-10.57)
P _{cat} ^d never vs. current alcohol use		0.83		0.22		0.27
P _{trend} ^b gram/day ^e		0.88		0.41		0.66

^a percentages may not add up to 100% due to missing data; ^b P value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables; ^c Among current and former smokers; ^d P value for difference in geometric mean concentration of OPG across categories of the exposure variable. Assessed using type III F statistics from linear regression models; ^e Among current drinkers; † Not applicable; Generalized linear models adjusted for: age (continuous), fasting status (<3, 3-6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing), and country.

Figure legend

Figure 1. Overview of participants from the parent case-control study who were eligible for the current study and in whom sRANKL and OPG concentrations were measured. Abbreviations: EPIC: European Prospective Investigation into Cancer and Nutrition; LLOD: Lower Limit of Detection; OPG: Osteoprotegerin; sRANKL: soluble Receptor Activator of Nuclear Factor κ B.

Figure 1.



Cancer Epidemiology, Biomarkers & Prevention

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Reproductive and lifestyle factors and circulating sRANKL and OPG concentrations in women: Results from the EPIC cohort

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